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Fingerprint analysis of the fruits of *Cnidium monnieri* extract by high-performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry

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Abstract

A method incorporating high-performance liquid chromatography (HPLC) with electrospray ionization (ESI) and tandem mass spectrometry (MS), with parallel analysis by HPLC with UV detection using a diode-array detector (DAD) was developed for the qualitative characterization of coumarin and chromone constituents in the fruits of *Cnidium monnieri*. The chromatographic separations were performed on a Diamonsil™ C18 column (4.6 mm × 200 mm, 5 μm) with water with 50 mM ammonium acetate and 2% acetic acid (A) and acetonitrile (B) as the mobile phase. According to the characteristic UV spectra, the information of molecular weight and structure provided by ESI–MS/MS, 13 coumarin and 7 chromone components were detected and identified. This method is rapid and reliable for identification of the constituents in the complex herbal system, and the fragmentation patterns proposed could be extended to the similar compounds.

Keywords: HPLC–DAD–ESI–MS/MS; Coumarin; The fruits of *Cnidium monnieri*; Fingerprint analysis

1. Introduction

Coumarins containing fused benzene and α-pyrone rings comprise a very large class of phenolic substances in plants [1]. Because of their wide applications in biology, their therapeutic activity (bacteriostatic, anticoagulant, spasmolytic, dermatological, and mutagenic), and their use in the identification of nucleic acids and in the food and hygiene industries, these compounds attract much interest [2–4]. Coumarins occur in over 100 plant families, for example, the Clusiaceae, Rutaceae, and Apiaceae. *Cnidii fructus* (she chuang zi), one of the widely used traditional Chinese medicines (TCM), is derived from mature and dry fruits of the Apiaceae plant *Cnidium monnieri* (L.) Cusson and has been used for treatment of pain in female genitalia, impotence and suppurative dermatitis and is soaked in rice wine as a tonic agent in China [5]. The fruit contains coumarins, chromones, essential oil, terpenoids and their glycosides. A number of coumarins, such as xanthotoxin, isopimpinellin, bergapten, imperatorin, osthole are present in this crude drug and regarded as active principle constituents (see Fig. 1.). Xanthotoxin is used orally or topically in combination with controlled exposure to long wavelength ultraviolet radiation (UVA) or sunlight to repigment vitiliginous skin in patients with idiopathic vitiligo [6]. Previous studies have shown that naturally occurring coumarins (e.g., imperatorin and isopimpinellin) inhibited P450-mediated enzyme activities *in vitro* [7]. Imperatorin and isopimpinellin also have the potential chemopreventive effects when administered in the diet [8]. The stimulation of melanogenesis by bergapten is related to increased tyrosinase synthesis. In addition, bergapten stimulated TRP-1 synthesis and induced a dose-dependent decrease of DCT activity without modification of protein expression [9]. Osthole could prevent postmenopausal osteoporosis [10]. It can also delay aging, build up strength, enhance immune function, and adjust sex hormone level. The ethanol extract of the fruits of *C. monnieri* and the five separate and recrystallized coumarins all have growth-inhibitory effects on the tumor cells [11].

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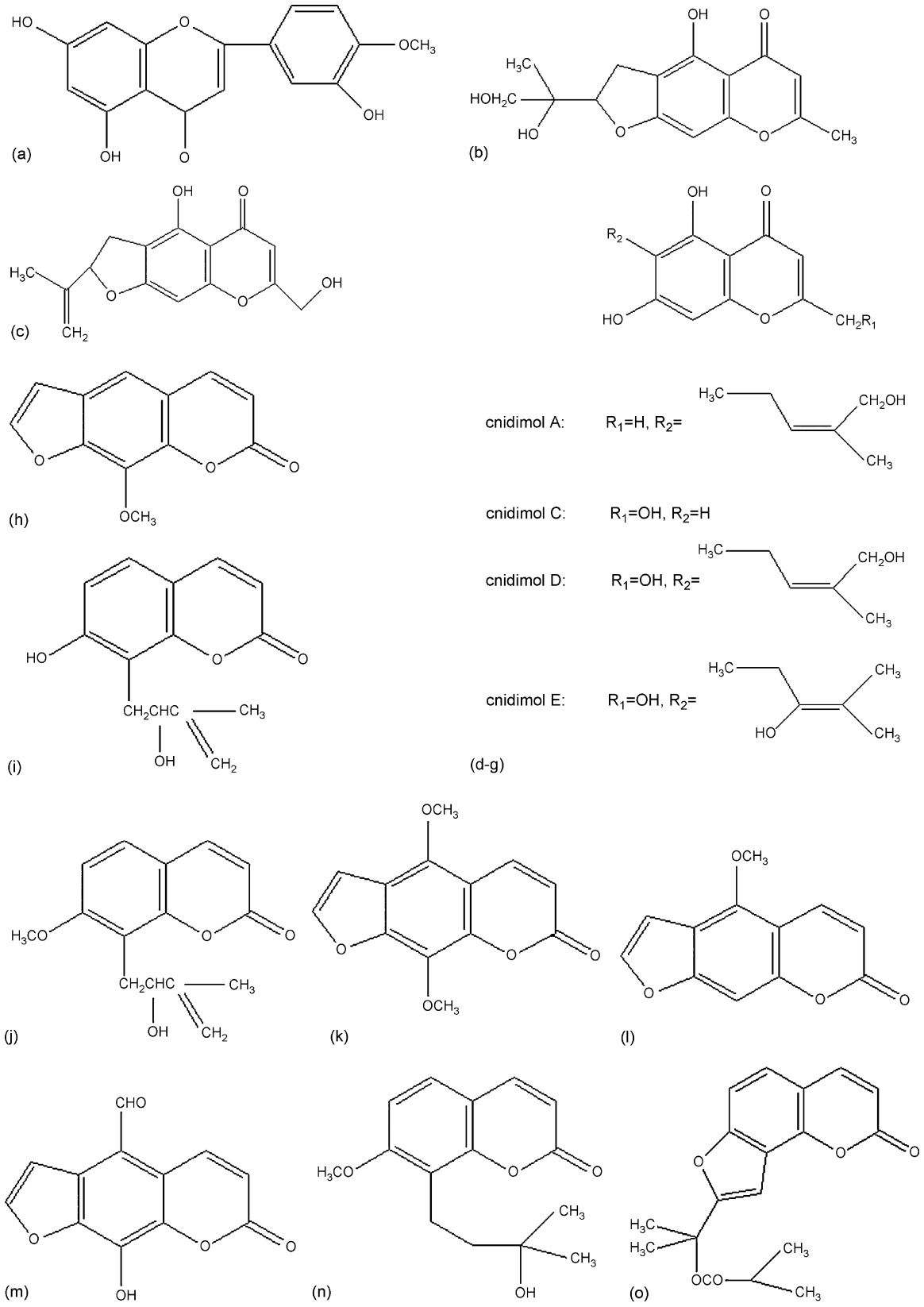


Fig. 1. Structures of the constituents identified from the fruits of *C. monnieri*. Chromones: (a) diosmetin (MW 300); (b) cnidimol B (MW 292); (c) DL-umtatin (MW 274); (d) cnidimol A (MW 292); (e) cnidimol E (MW 292); (f) cnidimol D (MW 292); and (g) cnidimol C (MW 208). Coumarins: (h) xanthotoxin (MW 216); (i) demethyl-auraptenol (MW 246); (j) auraptenol (MW 260); (k) isopimpinelline (MW 246); (l) bergapten (MW 216); (m) 5-formylxanthotoxol (MW 230); (n) 2′-hydrate-deoxymeranin (MW 262); (o) cnidiadin (MW 314); (p) oroselone (MW 226); (q) imperatorin (MW 270); (r) osthole (MW 244); (s) cniforin A (MW 375); and (t) edultin (MW 386). The structures of h, k, l, q, r were unambiguous, the others were tentatively identified.

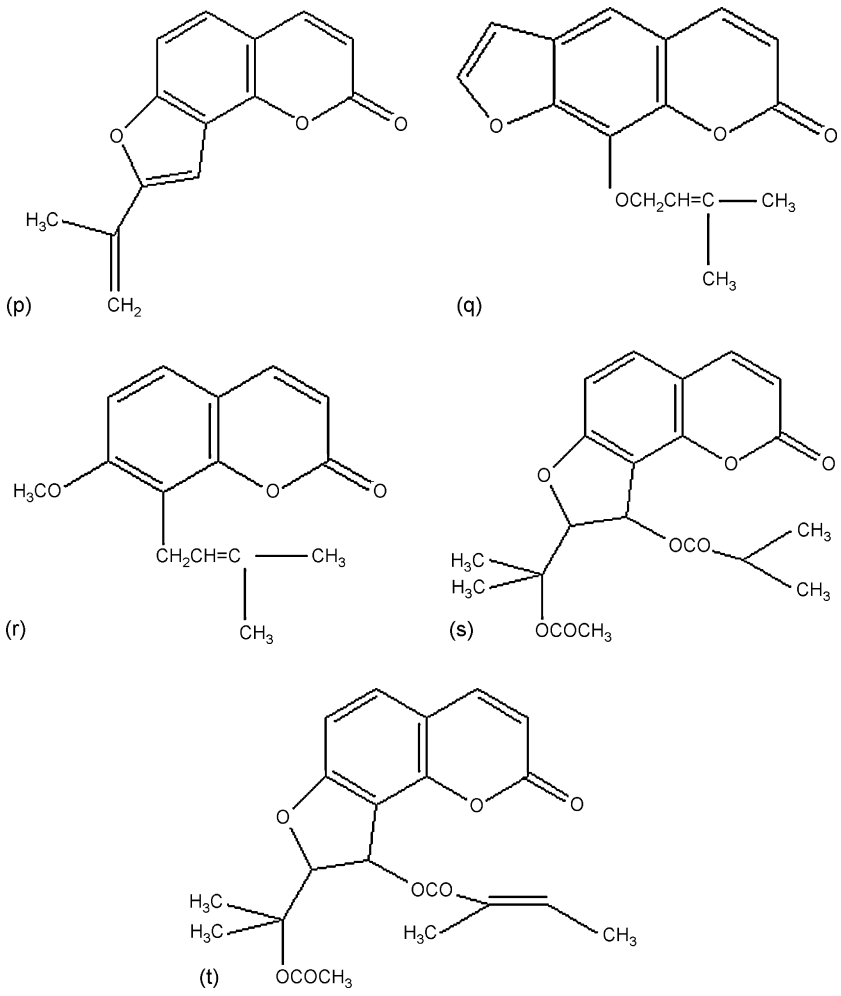


Fig. 1. (*Continued*).

Although the fruits of *C. monnieri* itself and the preparations containing its whole extracts were proved to be highly effective for the treatment of some diseases by the pharmacological studies and the clinical studies in China, they have not been officially recognized internationally. One important reason for this is the lack of an effective method to evaluate the quality of the raw medicinal materials. Establishment of the rapid and reliable analytical methods for multi-constituents in traditional Chinese medicines catches is increasingly in the focus of researchers' attention.

A variety of methods including high-performance liquid chromatography (HPLC) [12], gas chromatography (GC) [13], and micellar electrokinetic capillary chromatography (MEKC) [14] have been used for characterization of the coumarins in the fruits of *C. monnieri*. However, these separations only dealt with the five main coumarin components in the crude drug. It is obvious that they are not sufficient for authenticating this herb since perhaps all coumarin compounds are pharmacologically active components in the fruits of *C. monnieri*. Therefore, chemical characterization of the fruits of *C. monnieri* by using only the five main coumarins as the marker compounds seems to be insufficient, and not suitable for distinguishing the fruits of *C. monnieri* from its related Apiaceae herbs. In light of this, the characteristic fingerprint analysis is developed for this purpose.

Liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) is a significant analytical tool for the identification of the known compounds and helping the elucidation of the unknown compounds. ESI is generally used for molecular weight determination, and CID is performed to enhance fragmentation of the selected precursor ions. Based on fragmentation patterns, some unknown compounds or isomers in complex systems could be identified [15–25].

Recent success with the use of LC–ESI–MS/MS for characterizing complex plant extracts suggests that the technique might also be effective in comprehensive characterization of the multiple coumarin constituents in the complex herbal system. The report presented here details the establishment of an LC–DAD–ESI–MS/MS-based method that is capable of characterization of 13 coumarins and 7 chromones in one chromatographic run in the crude extract of the fruits of *C. monnieri* with the separation achieved both chromatographically and mass spectroscopically. This LC–DAD–ESI–MS/MS method enabled the simultaneous identification of the major bioactive constituents present in the fruits of *C. monnieri*, and can form the basis for the successful quality control of this medicinal herb.

2. Experimental

2.1. Materials and reagents

HPLC grade acetonitrile from Merck (Darmstadt, Germany) was used. Analytical grade methanol, ethanol, acetic acid, and ammonium acetate were purchased from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, China). Water for HPLC analysis was purified by a Milli-Q academic water purification system (Millipore, USA). Reference compounds, xanthotoxin, isopimpinellin, bergapten, imperatorin, osthole were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of these compounds was determined to be higher than 98% by normalization of the peak areas detected by HPLC. Their methanolic solutions were found to be very stable. *C. monnieri* fruits were collected from Xinyi of Jiangsu, and identified by Dr. Zhang.

2.2. Sample preparation

The fruits of *C. monnieri* were ground into powder, dried at 45 °C for 6 h. 1.0 g of the powder was ultrasonically extracted with 20 ml 95% ethanol in a conical flask for 1 h and then soaked at room temperature for 24 h. After filtering through a 0.45 μm membrane filter, the extract was transferred into a 100 ml volumetric flask, and adjusted to volume with 95% ethanol.

2.3. LC–DAD–ESI–MS/MS system

High-performance liquid chromatographic analysis was carried out using a Varian HPLC system (Palo Alto, CA, USA) equipped with a ProStar 410 autosampler, two ProStar 210 pumps with on-line degasser, column oven and a 335 diode array detector (DAD). The chromatographic conditions were: Diamonsil™ C18 column (4.6 mm × 200 mm, 5 μm, Dikma Technologies, Beijing, China); sample injection volume, 20 μl; the temperature of column oven, 30 °C; flow rate, 1.0 ml/min; mobile phases, water with 50 mM ammonium acetate and 2% acetic acid (A) and acetonitrile (B). A gradient programmer was used according to the following profile: 0–15 min, 35% B; 25–30 min linear increase to 80% B; 30–37 min hold on 80% B; 37–40 min linear decrease to 35% B. UV spectra were recorded from 210 nm to 500 nm, and the monitor wavelengths were set at 270 nm and 320 nm.

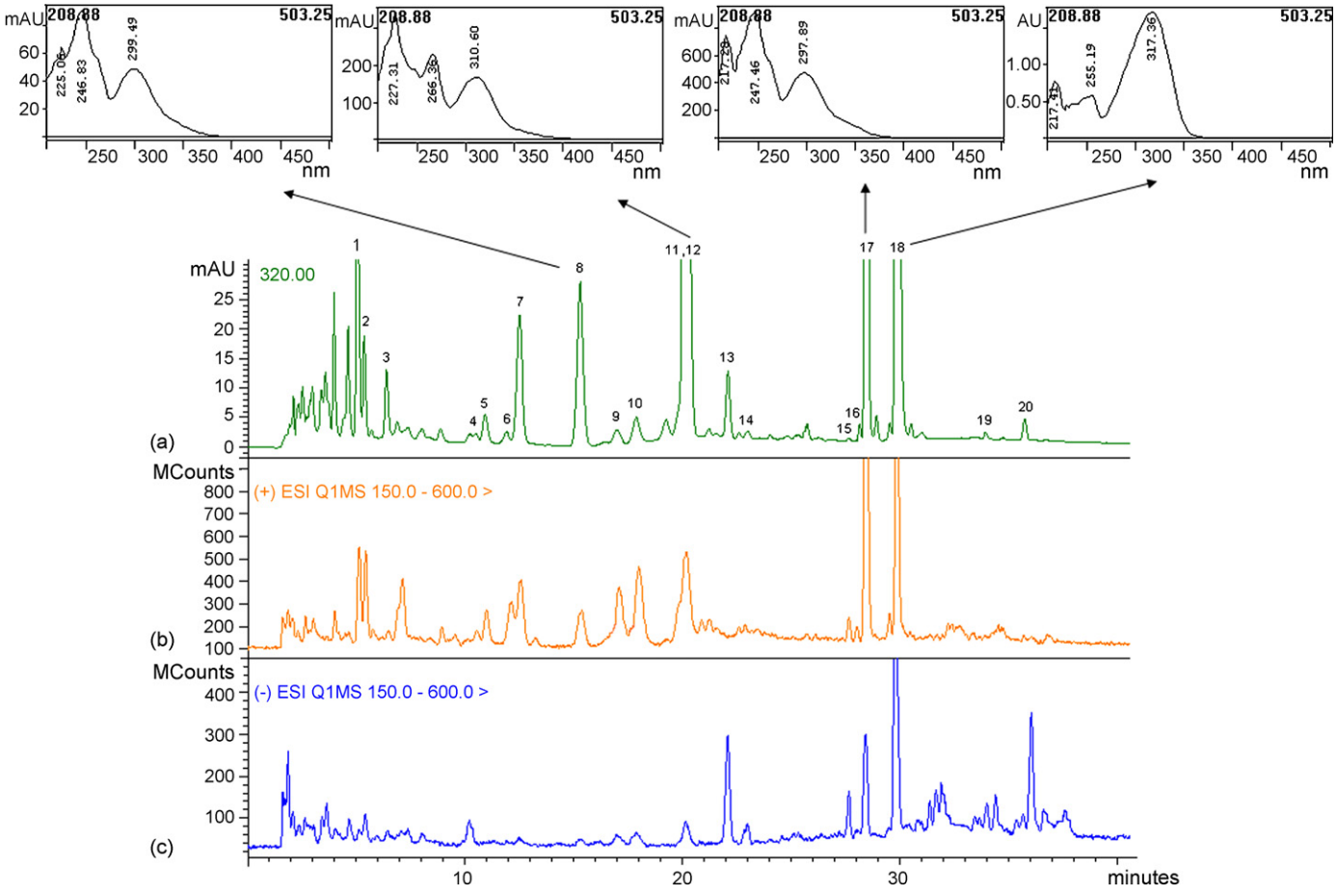
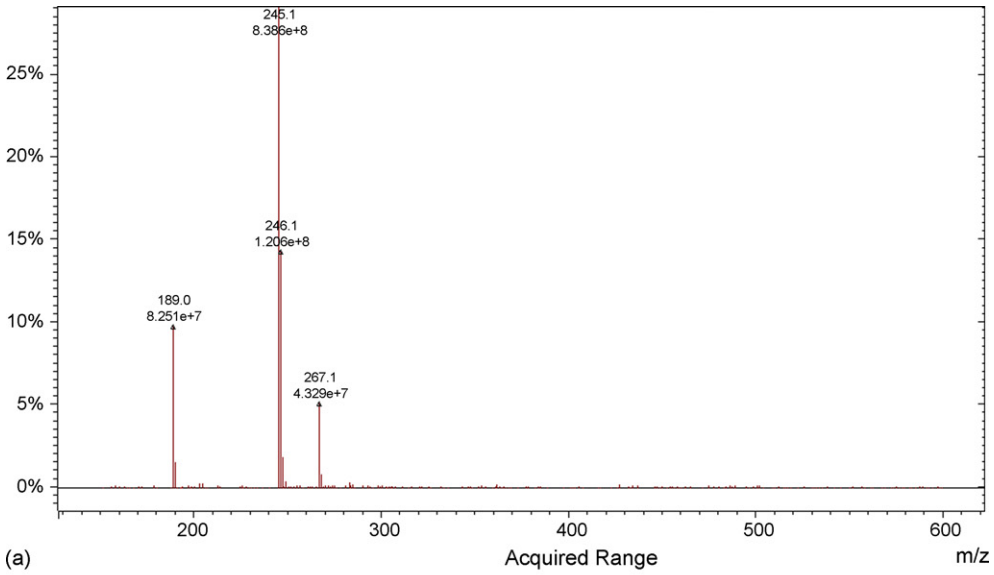
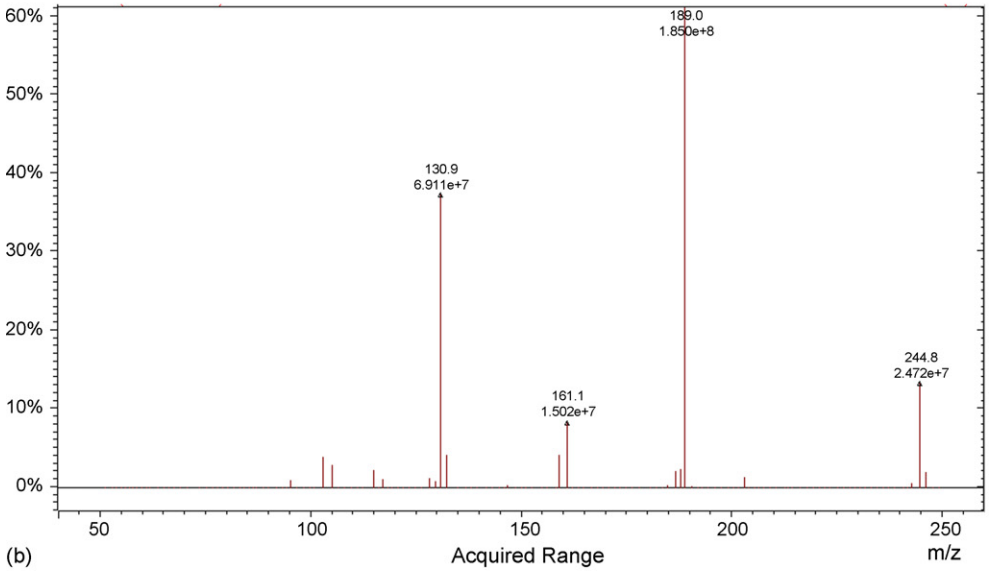


Fig. 2. Chromatograms of the fruits of *C. monnieri* with: (a) HPLC (320 nm); (b) MS–TIC (positive ion mode); and (c) MS–TIC (negative ion mode) detection. The peaks in chromatogram were identified by LC–DAD–MS/MS as follows: (1) diosmetin; (2) cnidimol B; (3) DL-umtatin; (4) cnidimol A; (5) cnidimol E; (6) cnidimol D; (7) cnidimol C; (8) xanthotoxin; (9) demethyl-auraptenol; (10) auraptenol; (11) isopimpinelline; (12) bergapten; (13) 5-formylxanthotoxol; (14) 2′-hydrate-deoxymeranin; (15) cnidiadin; (16) oroselone; (17) imperatorin; (18) osthole; (19) cniforin A; and (20) edultin.





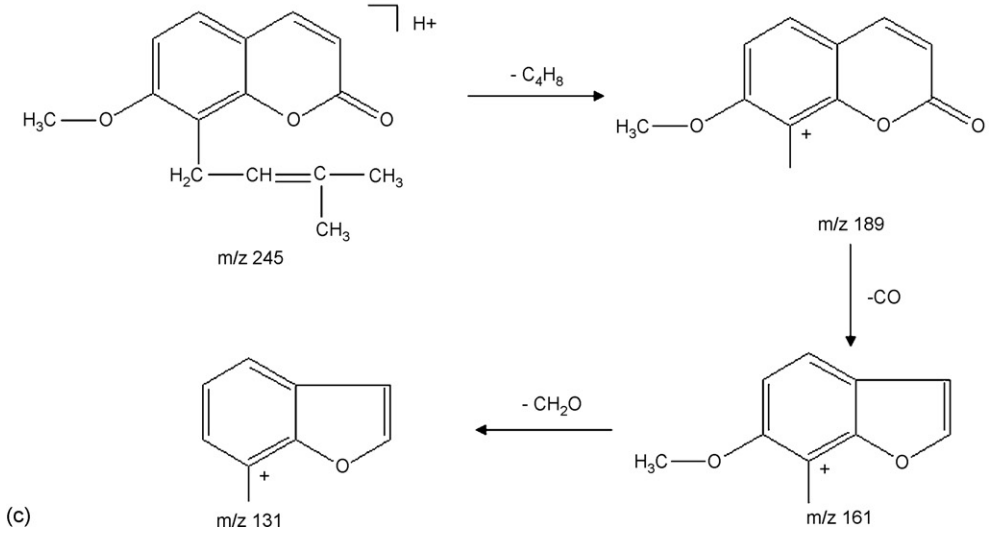


Fig. 3. MS (a) and MS/MS (b) spectra and the proposed fragmentation pattern (c) of osthole.

A 1200L electrospray tandem mass spectrometer (Palo Alto, CA, USA) equipped with an ESI interface was used for carrying out the MS and MS/MS analyses. Data were acquired and processed by Varian 1200L workstation software. The electrospray capillary potential was set to 35 V. Nitrogen was used as a drying gas for solvent evaporation. The API housing and drying gas temperatures were kept at 50 °C and 380 °C, respectively. Full scan data acquisition was performed from *m/z* 150 to 600 in both positive and negative MS scan mode. MS/MS experiments were performed by the collision of the precursor ions with helium gas. The collision energy values were automatically selected.

3. Results and discussion

3.1. Selection of extraction methods

The extraction efficiency of different methods including refluxing for 3 h, either in Soxhlet extractor or not, ultrasonication for 1 h, and soaking for 24 h at room temperature were investigated. Ultrasonication and soaking were found to be suitable for extracting the coumarin components completely. The extraction efficiency of various concentration of ethanol from 40% to 95% was also investigated. Most of the coumarin components in the herb can be extracted by 95% ethanol. On the basis of the above considerations, a two-step extraction procedure, ultrasonication with 95% ethanol for 1 h and then soaking for 24 h, was designed as the extraction method for the sample preparation of LC–MS/MS fingerprinting.

3.2. Optimization of HPLC conditions

Ammonium acetate and acetic acid were added to mobile phase A, to depress the tailing of the peaks of phenolic compounds. Concentrations of 50 mM ammonium acetate and 2% acetic acid were selected to ensure the reproducibility of the fingerprint chromatogram. Since isocratic elution was not suitable

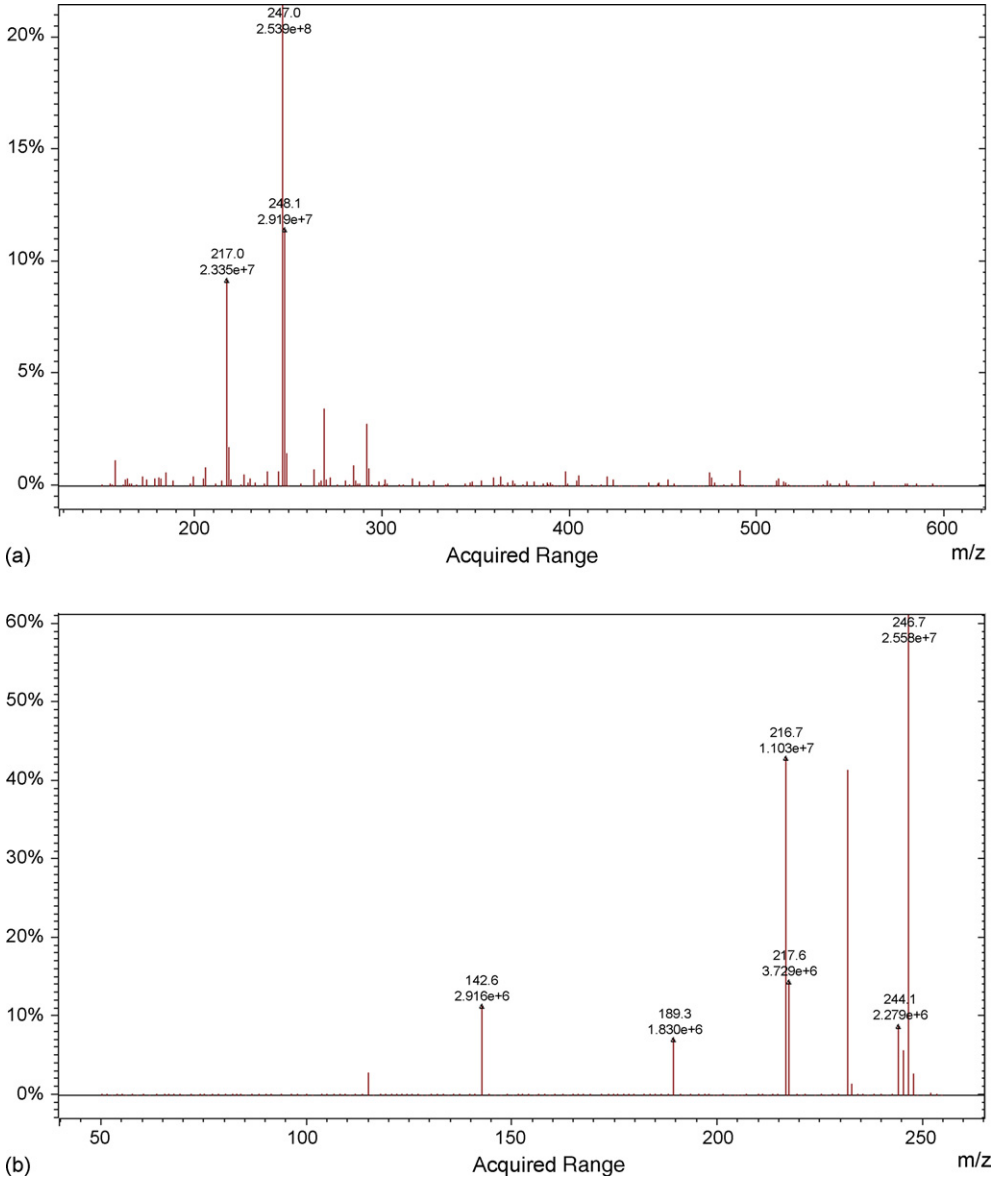


Fig. 4. MS (a) and MS/MS (b) spectra and the proposed fragmentation pattern (c) of isopimpinelline.

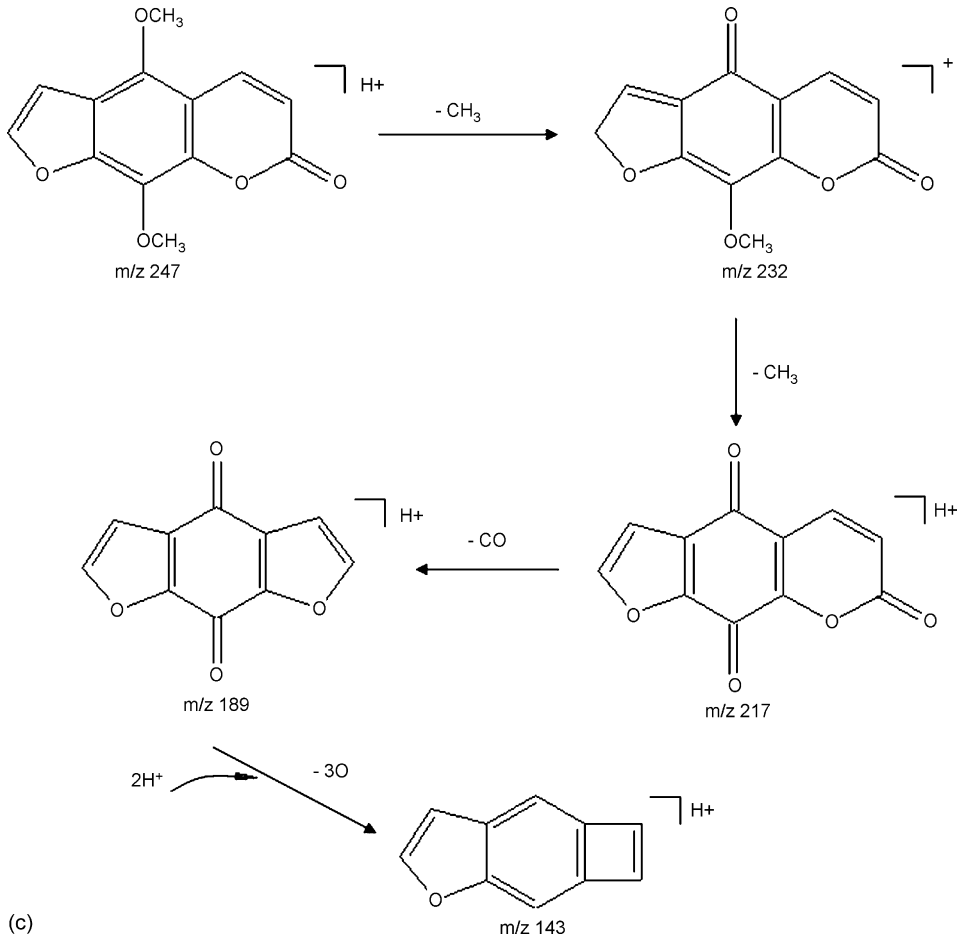


Fig. 4. (*Continued*).

Table 1
HPLC–DAD–ESI–MS data and identification of constituents from the fruits of *C. monnieri*

Peak	t_R (min)	λ_{max} (nm)	Ion mode	Molecular ion and main fragments	Identification
1	5.075	226, 255, 321	Positive	301, 324, 339, 152, 132, 124	Diosmetin
2	5.379	212, 227, 319	Positive	293, 217, 253	Cnidimol B
3	6.400	212, 225, 248, 264, 305	Positive	275, 297, 313, 233, 203	DL-umtatin
4	10.232	210, 221, 232, 253, 295	Negative	291, 189	Cnidimol A
5	10.936	214, 228, 279, 319	Positive	293, 315, 276, 222	Cnidimol E
6	11.909	219, 227, 316	Positive	293, 315, 276, 262, 260, 222	Cnidimol D
7	12.507	213, 220, 256, 321	Positive	209, 153	Cnidimol C
8	15.296	225, 247, 299	Positive	217, 239, 255, 202, 174, 131	Xanthotoxin
9	16.995	213, 221, 229, 280, 322	Positive	247, 177, 149	Demethyl-auraptenol
10	17.883	221, 321	Positive	261, 191, 163, 133	Auraptenol
11	20.192	227, 266, 311	Positive	247, 269, 285, 232, 217, 189, 143	Isopimpinelline
12	20.518	221, 250, 258, 310	Positive	217, 202, 188, 174, 159, 132	Bergapten
13	22.104	218, 230, 322	Negative	229, 172	5-Formylxanthotoxol
14	22.995	228, 289, 338	Negative	261, 245, 217, 186	2′-Hydrate-deoxymeranin
15	27.645	220, 298	Negative	313, 349, 373, 243	Cnidiadin
16	28.013	213, 277	Positive	227, 199	Oroselone
17	28.461	217, 247, 298	Positive	271, 293, 309, 563, 203, 69	Imperatorin
18	29.885	217, 255, 317	Positive	245, 267, 189, 161, 131	Osthole
19	33.939	237, 256, 291	Negative	374, 433, 331, 303, 243	Cniforin A
20	35.731	248, 297	Negative	445, 343, 303, 243	Edultin

for the complete separation of coumarins in the fruits of *C. monnieri*, two sections of slopes were built into the gradient so that it covered the parts of the chromatogram where increased resolution is desired. This gradient of the mobile phase could achieve maximum throughput and optimal resolution. The wavelength for the detection of the components in the fruits of *C. monnieri* was selected by using a DAD detector. It was found that the chromatograms at 270 nm and 320 nm could well represent the profile of the constituents. The representative chromatogram at 320 nm is shown in Fig. 2a. The typical on-line UV–vis spectra of four respective coumarins, xanthotoxin, bergapten, imperatorin, and osthole, recorded using a DAD detector is also shown.

3.3. HPLC–MS/MS analysis

The MS spectra were detected in both the positive and negative ion mode and their TIC chromatograms are shown in Fig. 2b and c, respectively. In MS spectra, most of the constituents exhibited their quasi-molecular ions $[M+H]^+$, adduct ions $[M+Na]^+$ and $[M+K]^+$ in positive ion mode, while exhibited $[M-H]^-$, $[M+Cl]^-$, and $[M+CH_3COO]^-$ in negative ion mode. The positive ion mode was found to be more sensitive and of lower noise to the most coumarins in the fruits of *C. monnieri*. Based on the *m/z* value, UV spectra and the comparison with standard compounds, five peaks were unambiguously identified as xanthotoxin (8), isopimpinelline (11), bergapten (12), imperatorin (17), and osthole (18). Other 15 peaks were tentatively identified as diosmetin (1), cnidimol B (2), DL-umtatin (3), cnidimol A (4), cnidimol E (5), cnidimol D (6), cnidimol C (7), demethyl-auraptenol (9), auraptenol (10), 5-formylxanthotoxol (13), 2′-hydrate-deoxymeranin (14), cnidiadin (15), oroselone (16), cniforin A (19), and edultin (20) by comparing their *m/z* value and UV spectra with the literature data [26–34]. The results are listed in Table 1 and the structures of these compounds are

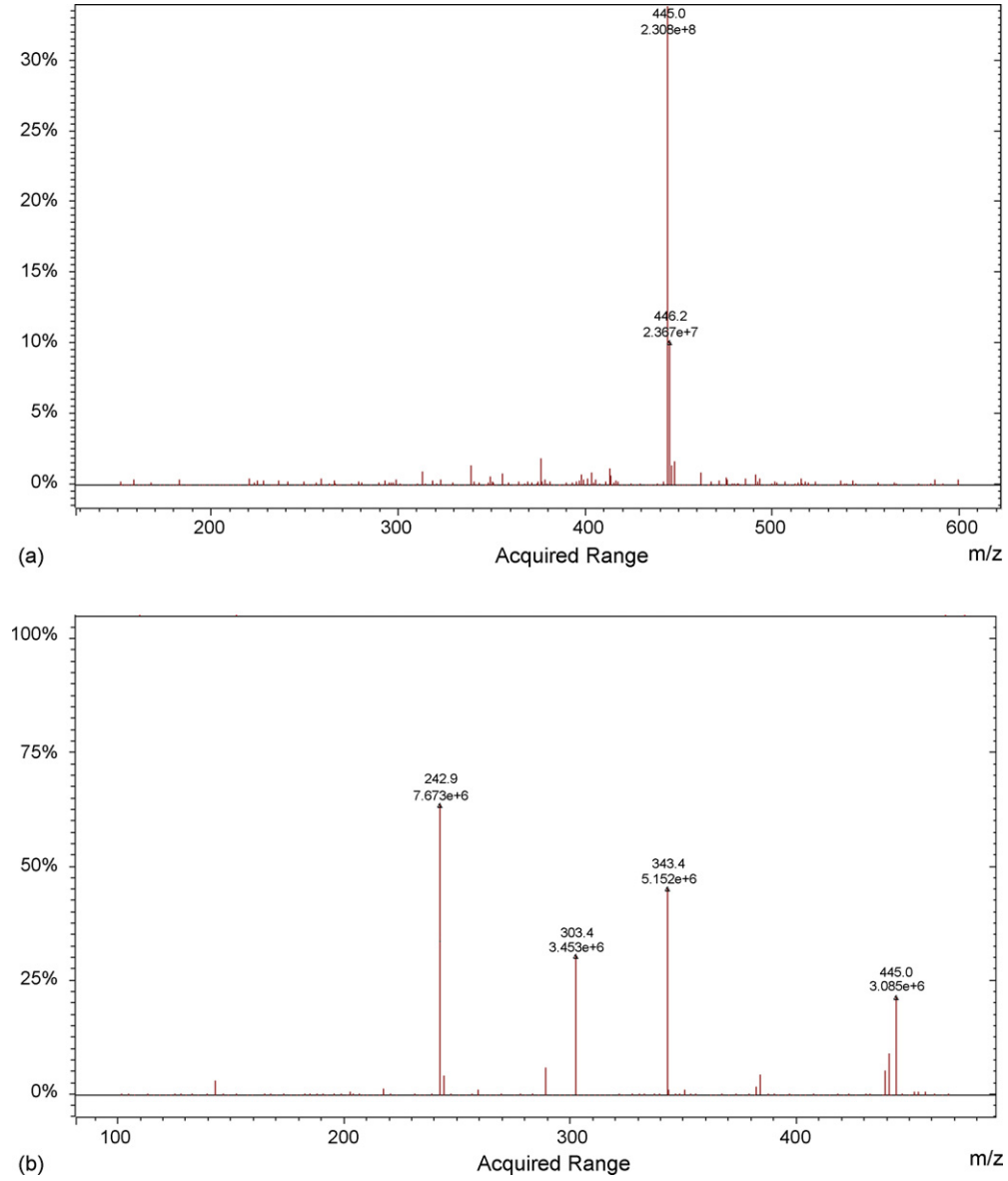


Fig. 5. MS (a) and MS/MS (b) spectra and the proposed fragmentation pattern (c) of edultin.

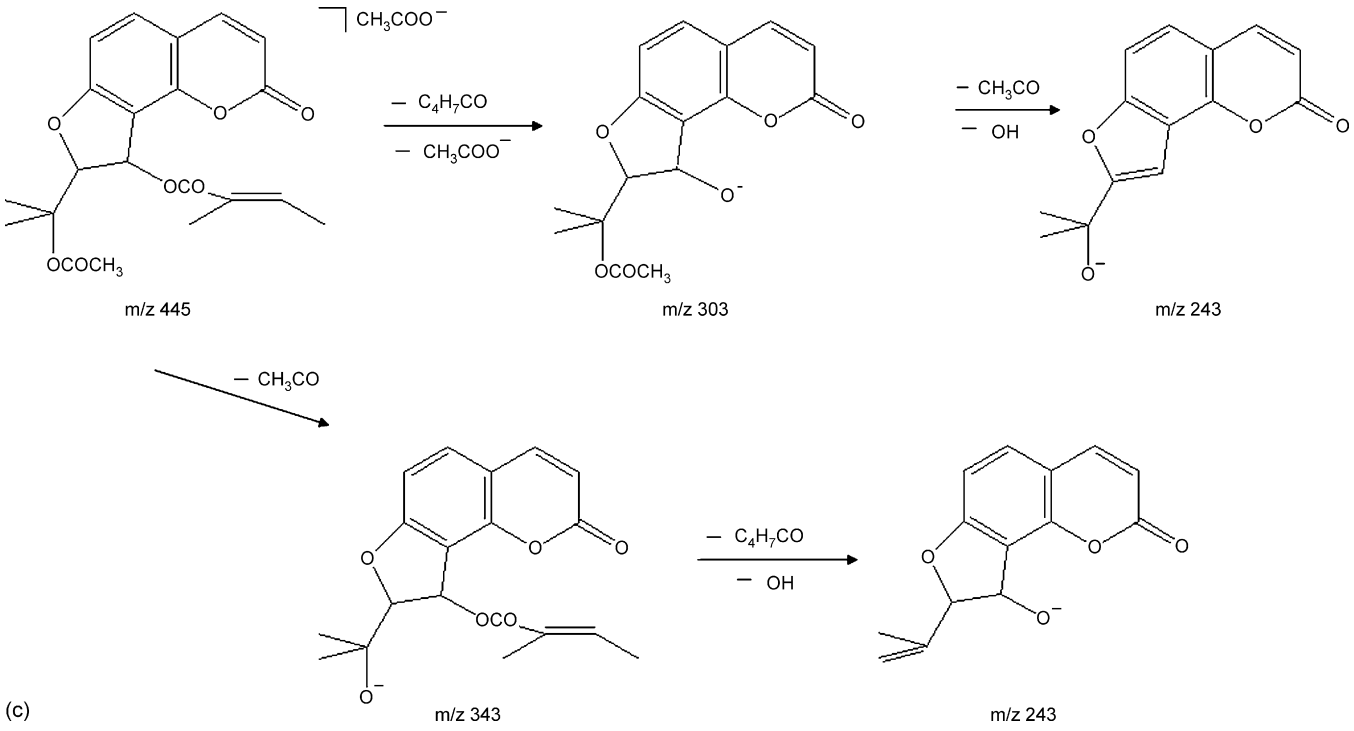


Fig. 5. (*Continued*).

shown in Fig. 1. It could be seen that the constituents in the fruits of *C. monnieri* detected in these assay were coumarins (8–20) and chromones (1–7), while the types of the coumarins included simple coumarin (9, 10, 18), linear furocoumarin (8, 11–14, 17), and angular furocoumarin (15, 16, 19, 20).

Fig. 3 shows the MS and MS/MS spectra and the proposed fragmentation pattern of peak 18, a simple coumarin. The MS spectra in the positive mode exhibited an abundant parent ion $[M+H]^+$ at *m/z* 245 and ion $[M+Na]^+$ at *m/z* 267, while a fragment ion at *m/z* 189 was also observed. By the optimum cone voltage setting at 20 eV, the fragment ion at *m/z* 189 could be attributed to the loss of C_4H_8 from the parent ion. This was further fragmented to yield an ion with *m/z* 161, signaling loss of CO. Subsequent loss of 30 mass units equating to the loss of CH_2O occurred with a peak observed at *m/z* 131.

A fragmentation pathway of linear furocoumarin was presented by the fragmentation pattern of peak 11. An abundant parent ion $[M+H]^+$ at *m/z* 247 and ion $[M+Na]^+$ at *m/z* 269 were produced. This parent ion fragmented initially with twice loss of methyl (*m/z* 232 and *m/z* 217), followed by loss of 28 mass units attributed to CO (*m/z* 189), and finally generated a peak at *m/z* 143 by loss of 46 mass units (see Fig. 4.).

For the angular furocoumarin, such as peak 20, once again a parent ion $[M+CH_3COO]^-$ at *m/z* 445 was observed in the negative mode, which dissociated in MS/MS to generate ions at *m/z* 343, 303 and 243. A loss of C_4H_7CO and CH_3CO was observed in no sequence (see Fig. 5.).

4. Conclusion

Combining the HPLC separation, the UV absorption pattern and the information of molecular weight and structure provided by ESI–MS/MS, HPLC–DAD–ESI–MS/MS was proved to be an effective tool for the study of a complex herbal system. This technique showed the superiority and became a key strategy in analyzing traditional Chinese medicines.

According to the principle to choose the relevant marker compounds, which are specific to species, taxonomic evidences of stability, specificity, discontinuity and limitation, integrality and correlative differences of the marker compounds were significant. The results in our study showed that thirteen coumarins including simple coumarin, linear furocoumarin, and angular furocoumarin and seven chromones were coexistent in the fruits of *C. monnieri*. Although some other plants, especially in Apiaceae, and Rutaceae, may have more or less coumarins and chromones as in the fruits of *C. monnieri*, no report was found on the simultaneous occurrence of the three types of coumarins and the chromones in other plants. For example, ostheol, bergapten, xanthotoxin, isopimpinellin, and imperatorin were all present in the roots of *Heracleum rapula* [46], while the chromones were not found. This is a significant characteristic in the fingerprint chromatogram of the fruits of *C. monnieri*. Then the other tentatively identified coumarins and chromones in the fingerprint chromatogram can assist to identify the plant species. The compounds in other related plants are shown in Table 2 [35–53].

In this study, the chemical constituents in the fruits of *C. monnieri* were analyzed by HPLC–DAD–ESI–MS/MS. Analyzed by direct infusion, the fragmentation patterns of reference compounds were proposed. Based on the fragmentation patterns and the comparison of the UV, MS data of sample with the published literature data, twenty constituents in the fruits of *C. monnieri* were identified. Among them, five main coumarins were unequivocally and the others tentatively identified. The fin-

Table 2
Distribution of the coumarins and chromones in the representative plants of Apiaceae and Rutaceae

Plant	Coumarin						Chromone	Reference
	Simple coumarin (osthole)	Linear furocoumarin				Angular furocoumarin		
		Xanthotoxin	Isopimpinelline	Bergapten	Imperatorin			
Angelica dahurica	√	√		√	√			[35,36]
Angelica pubescence	√	√		√		√		[37,38]
Angelica morri					√		√	[39,40]
C. monnieri	√	√	√	√	√	√	√	[41]
Cnidium dahuricum						√		[42]
Peucedanum ostruthium	√				√			[43,44]
Peucedanum acaule	√					√		[45]
H. rapula	√	√	√	√	√			[46]
Heracleum stenopterum			√	√		√		[47]
Saponikvia cata		√		√	√		√	[48]
Bupleurum chinese							√	[49]
Murraya siamensis						√		[50]
Murraya exotica	√							[51]
Zanthoxylum dimorphophyllum			√			√		[52]
Zanthoxylum shinifolium				√		√		[53]

gerprint chromatograms can be useful to ensure the safety and efficacy, and to optimize the quality control of the fruits of *C. monnieri*.

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